Immunology 1990 **71** 133–138

Immunization of guinea-pigs against *Rhipicephalus appendiculatus* adult ticks using homogenates from unfed immature ticks

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Accepted for publication 12 April 1990

SUMMARY

Guinea-pigs immunized with homogenates of unfed larvae and nymphs of the tick *Rhipicephalus appendiculatus* developed significant levels of protective immunity to infestation with adults of this species. The mean engorged weight of female ticks feeding on immunized animals (181·96 ± 05·63 mg and 170·11 ± 11·54 mg) was reduced by an average of 46% and 51%, respectively, compared to that of female ticks feeding on control guinea-pigs, although in some individual animals the reduction was as high as 86%; the mean egg mass weight was also significantly reduced. Electrophoretic separation of the homogenates followed by immunostaining with post-infestation sera revealed several antigen bands common to all stages. Two bands of 36,500 and 23,000 molecular weight (MW) were recognized in all homogenates by post-adult infestation serum, but not by post-larval or post-nymphal infestation sera, suggesting that these may be antigens specifically involved in feeding by adult ticks, and are either not presented to the host's immune system or presented only in minimal amounts during feeding by immature stages. Sera from animals immunized with the homogenates did not recognize either of these antigens. Post-immunization sera did, however, stain two bands of 84,000 and 60,000 MW in the homogenates which were not recognized by post-infestation sera.

INTRODUCTION

Recent work in Australia (Johnston, Kemp & Pearson, 1986; Opdebeeck *et al.*, 1988; Kemp *et al.*, 1989) has demonstrated the feasibility of successful immunization of cattle against the 1-host cattle tick *Boophilus microplus*, and purified and characterized one of the protective antigens from fed female ticks (Willadsen *et al.*, 1989). In Africa, one of the most important ectoparasites of cattle is the 3-host tick *Rhipicephalus appendiculatus*, the vector of East Coast fever. The high cost of chemical control of ticks is beyond the financial resources of many tropical developing countries and host immunization offers an attractive and potential alternative for tick infestation control. African cattle acquire resistance to natural infestations with *R. appendiculatus* (Rechav, 1987). Mongi *et al.*, (1986), Jongejan *et al.*, (1989) and Nyindo, Essuman & Dhadialla (1989) have shown that animals can also be immunized against adult *R. appendiculatus* by inoculating them with whole adult extracts or midgut or salivary gland extracts from female ticks. We now report the immunization of animals against adult *R. appendiculatus* with homogenates from unfed immature stages of this species, and the demonstration of shared antigens between larvae, nymphs and adults.

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MATERIALS AND METHODS

Preparation of unfed tick homogenates

Larvae and nymphs of *R. appendiculatus*, approximately 2 weeks old, from a laboratory colony, were killed by chilling at −20° and homogenized in cold phosphate-buffered saline (PBS; Dulbecco's A, Oxoid, Basingstoke, Hants), pH 7·2, to give an approximate 10% (w/v) suspension. The suspensions were centrifuged at 12,000 ***g*** for 1 hr at 4° and the supernates filtered through 0·22 μm filters and stored at −70° until used. Protein content of the homogenates was determined by the method of Lowry *et al.* (1951).

Immunization of guinea-pigs

Twenty-one larval and eight nymphal homogenates were prepared and tested separately on groups of two to three female guinea-pigs (Dunkin/Hartley strain; A. Tuck & Son, Battlesbridge, U.K.) weighing 500–600 g. Each animal was inoculated subcutaneously with the homogenate emulsified with an equal volume of Freunds' incomplete adjuvant (FIA) at the rate of 3 mg protein per kg body weight. A second similar inoculation was given 2 weeks later. In experiments with 12 of the larval homogenates and with the nymphal homogenates, the animals received a third inoculation with antigen only, 3–4 weeks after the first. Four to 5 weeks (depending on whether the animals were inoculated twice or three times) after the first inoculation, 10 female and 15 male *R. appendiculatus* ticks, 4–8 weeks old, were released into a cloth feeding bag fixed to the back of each

Table 1. Weights of *R. appendiculatus* female ticks and their egg masses fed on guinea-pigs immunized with larval and nymphal homogenates and on naive guinea-pigs

	Immunized mean ± SEM (mg) (range)	Control mean ± SEM (mg) (range)	% reduction	*P*
Larval				
Female engorged weight	181·96 ± 05·63 (*n* = 415) (79·95 ± 12·25–249·33 ± 20·25)	338·00 ± 07·04 (*n* = 251) (277·75 ± 24·85–405·32 ± 29·88)	46	< 0·001
Egg mass weight	71·52 ± 05·08 (*n* = 288) (26·44 ± 05·83–115·48 ± 09·36)	127·87 ± 05·75 (*n* = 195) (70·56 ± 17·40–161·23 ± 10·14)	44	< 0·001
Lymphal				
Female engorged weight	170·11 ± 11·54 (*n* = 133) (48·24 ± 06·71–306·35 ± 38·49)	349·50 ± 12·36 (*n* = 137) (264·20 ± 23·62–443·08 ± 29·37)	51	< 0·001
Egg mass weight	83·48 ± 11·99 (*n* = 68) (56·19 ± 11·85–138·53 ± 22·34)	145·80 ± 13·03 (*n* = 88) (111·61 ± 08·06–190·51 ± 27·25)	43	< 0·01

guinea-pig. Guinea-pigs of similar weights and numbers inoculated with FIA only served as controls and were challenged with the same number of ticks (of the same age and from the same batches) as on the immunized groups. Engorged female ticks after dropping from the hosts were weighed, individually tubed and kept at 25 ± 1°, 80 ± 5% relative humidity and a 12 hr photoperiod for egg laying. At cessation of oviposition, the egg mass from each tick was weighed and on completion of larval eclosion the hatch rate was scored for each egg mass.

Data analysis

Differences in weights of engorged female ticks, egg masses, conversion efficiency index (CEI), which is a measure of the ability of the ticks to convert blood meal to eggs expressed as the percentage weight of the egg mass to female engorged weight, and average feeding times of the ticks from immunized and control groups were tested for significance using the Student's *t*-test. Differences in the number of fed ticks recovered, of ovipositing female ticks and of fed ticks in the same weight categories in immunized and control groups were tested for significance using the chi-squared test. A significant ($P < 0·05$) difference in at least one of these parameters was taken as evidence of development of protective immunity.

Chromatography

Twenty-microlitre samples of the homogenates were loaded on to a 300 mm × 7·5 mm TSK G3000SW size exclusion column (Toya Soda, Japan, supplied by Beckman Instruments, High Wycombe, Bucks, U.K.) fitted with a TSK SW guard column connected to a Beckman HPLC system. The samples were eluted in PBS at a flow rate of 0·8 ml per minute. Molecular weights (MW) of the eluted protein peaks were estimated by comparison with the elution times of protein standards of known MW (Bio-Rad, Hemel Hempstead, Herts).

Polyacrylamide gel electrophoresis (PAGE)

Homogenates of unfed larvae, nymphs and adults (prepared from equal numbers of male and female ticks in a manner similar to that for larvae and nymphs) were electrophoretically separated on SDS–PAGE gels, containing 12% acrylamide, using the method of Laemmli (1970). Homogenates and MW marker proteins were boiled for 2 min in SDS sample buffer containing 5% 2-mercaptoethanol, before loading on to gels. The gels were electrophoresed at 160 V for 5 hr.

Western blotting

Electrophoretic transfer of the proteins from gel to nitrocellulose paper (NCP; Schleicher and Schuell, Dassel, FRG) was accomplished by the method of Burnette (1981) and were blotted overnight at 30 V. The blots (NCP) were then cut into strips corresponding to the lanes on the gels and quenched in PBS-Tween 20 (PBST) buffer, pH 7·6. Unbound sites on sample lanes were blocked by incubation with 1% casein in PBST. Polyclonal sera from guinea-pigs after two sequential larval infestations (anti-larval serum), two sequential nymphal infestations (anti-nymphal serum) and two sequential adult infestations with *R. appendiculatus* (anti-adult serum) were used for immunostaining. Serum from a naive guinea-pig was used as a control. The NCP strips were incubated for 90 min in the appropriate serum (diluted 1:200 in PBST) and thoroughly washed with PBST before incubation for 1 hr with rabbit anti-guinea pig whole immunoglobulins (Dako Ltd, High Wycombe, Bucks, U.K.) conjugated with horseradish peroxidase (HRP), diluted 1:1000 in PBST. After thorough washing with PBST, the strips were incubated in peroxidase substrate buffer (0·3 g/l of 3,3-diaminobenzidine tetrachloride in PBS containing 60 μl of 6% hydrogen peroxide) until the reaction was complete, washed with PBS and the strips dried between sheets of blotting paper.

RESULTS

Immunization with larval homogenates

There was no significant difference in the number of fed *R. appendiculatus* females recovered from each of the guinea-pigs in the immunized and control groups. The feeding time (mean ± SEM) of females on immunized guinea-pigs was shorter, 7·9 ± 0·3 days, than on the control animals (8·6 ± 0·3 days). Although the difference was not significant, the pattern was consistent for each experiment.

Nineteen of the 21 homogenates gave significant levels of protective immunity, as judged by the reduction in engorged weight of female ticks feeding on the immunized animals compared to those from the controls (Table 1). The reduction in weight ranged from 35% to as high as 73%. The mean engorged weight of female ticks from all the control guinea-pigs was 338·00 ± 07·04 mg and from all immunized guinea-pigs

Table 2. Distribution of *R. appendiculatus* engorged female ticks according to weight categories from guinea-pigs immunized with larval and nymphal homogenates and from naive guinea-pigs

Weight category (mg)	Number of ticks			
	Larval homogenate		Nymphal homogenate	
	Immunized ($n=415$)	Control ($n=251$)	Immunized ($n=133$)	Control ($n=137$)
1–49	50	5	39	7
50–99	80	7	15	3
100–149	63	7	12	2
150–199	50	5	12*	10
200–249	41*	17	15*	7
250–299	49*	34	13*	16
300–349	44	51	10*	20
350–399	26	56	12	25
400–449	9	35	2	10
450–499	3	16	2	17
500+	0	18	1	20

* Not significantly ($P>0{\cdot}05$) different from controls.

181·96 ± 05·63 mg (46% mean reduction, $P<0{\cdot}001$). A significantly higher proportion of ticks from the immunized group weighed less than 200 mg and a significantly higher proportion in the control group weighed over 300 mg (Table 2). The reductions in engorged weight obtained on each of the 48 immunized guinea pigs are given in Table 3 and show that individual animals varied in the degree of protective immunity achieved.

Two inoculations (eight homogenates tested) provided similar protection to guinea-pigs as three inoculations (12 homogenates tested). Female ticks from the group receiving two inoculations weighed 194·12 ± 08·84 mg and those from the three-times inoculated group weighed 174·37 ± 07·27 mg.

Mean egg mass weight from ticks in the immunized group (71·52 ± 05·08 mg) was reduced by 44% ($P<0{\cdot}001$), compared to the mean egg mass weight (127·87 ± 05·75 mg) from those in the control group (Table 1). A higher proportion (195 out of 251) of ticks feeding on the control guinea-pigs laid eggs compared to those (257 out of 379) feeding on guinea-pigs that had developed protective immunity ($P<0{\cdot}01$).

Immunization did not appear to have any significant effect on the conversion efficiency index (CEI) or on the hatch rate of eggs laid by the female ticks (data not presented).

Immunization with nymphal homogenates

The results were very similar to those obtained with the larval homogenates (Table 1). Six of the eight homogenates gave significant protection, which resulted in reductions in engorged weight ranging from 39% to as high as 82%. The mean weight of female ticks from all inoculated guinea-pigs was 170·11 ± 11·54 mg and from all control guinea-pigs 349·50 ± 12·36 mg, giving a reduction of 51% ($P<0{\cdot}001$). A significantly higher proportion of ticks in the immunized group weighed less than 150 mg and a significantly higher proportion in the control group weighed over 350 mg (Table 2). The reductions in engorged weight obtained on each of the 18 immunized animals are shown in Table 4. As with the larval homogenates, there was some variation between individual animals in the level of protective immunity.

The mean egg mass weight from all female ticks in the immunized group (83·48 ± 11·99 mg) was reduced by 43%

Table 3. Percentage reduction of *R. appendiculatus* female engorged weight from individual guinea-pigs immunized with larval homogenates

Homogenate no.	% reduction on individual animals	Mean
1	63*	63
2	35–56	45
3	34–44	39
4	33–53	44
5	67–79	73
6	4†–49	26†
7	26†–81	50
8	55–63	60
9	31–57	43
10	44–45	45
11	13†–21†	18†
12	35–36	35
13	43–44	43
14	43–60–78	57
15	14†–56–58	42
16	16†–39–73	42
17	34–39–73	49
18	49–73	58
19	61–62	62
20	60*	60
21	31–63–70	50
	22†–40–46	35

† Weight reduction not significant ($P>0{\cdot}05$) compared to controls.

* One animal out of two died before challenge feed by adult ticks.

Table 4. Percentage reduction of *R. appendiculatus* female engorged weight from individual guinea-pigs immunized with nymphal homogenates

Homogenate no.	% reduction on individual animals	Mean
1	52–65	59
2	33–69	47
3	28†–52	39
4	57–66–86	69
5	77–84–85	82
6	33–75	56
7	8†–31†	20†
8	24†–51†	36†

† Weight reduction not significant ($P>0{\cdot}05$) compared to controls.

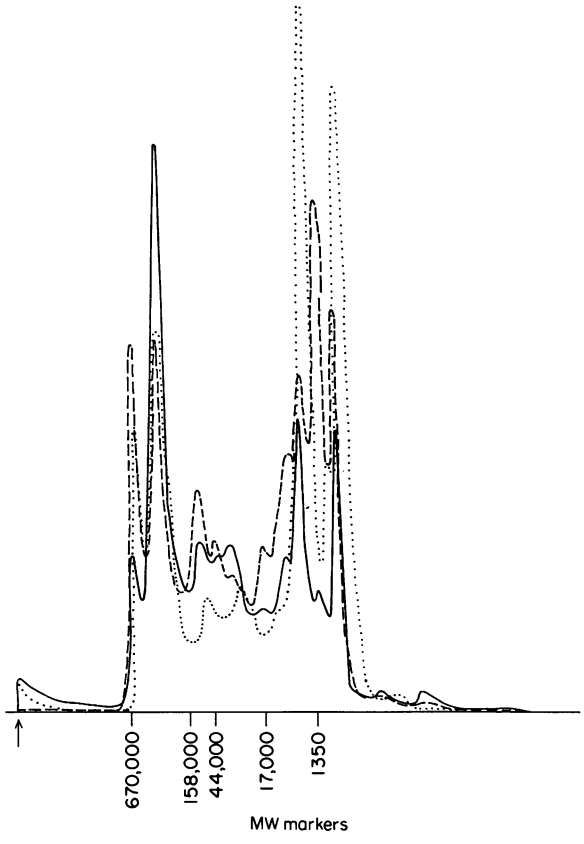

Figure 1. Protein elution profiles of unfed larval (· · ·), nymphal (— — —) and adult (——) *R. appendiculatus* homogenates. Arrow indicates injection of samples. Chromatographic conditions were: column, TSK G3000SW 300 mm × 7·5 mm internal diameter (i.d.); flow rate, 0·8 ml/min; eluent PBS pH 7·2; MW standards vitamin B 12 (1350), myoglobin (horse) (17,000), ovalbumin (chicken) (44,000), gamma-globulin (bovine) (158,000), thyroglobin (bovine) (670,000).

($P < 0{\cdot}01$) compared to the mean egg mass weight (145·80 ± 13·03 mg) from females in the control group (Table 1). A higher proportion (78 out of 83) of ticks feeding on the control guinea-pigs laid eggs compared to those (60 out of 89) feeding on guinea-pigs that developed protective immunity ($P < 0{\cdot}001$).

There was no reduction in the CEI of female ticks feeding on the immunized animals; neither did immunization affect the hatch rate of eggs (data not published).

Size exclusion HPLC of crude unfed homogenates

Chromatograms of the protein elution profiles of unfed larval, nymphal and adult homogenates are shown in Fig. 1. Eight to 10 major peaks were seen and there was a close fit between the elution profiles of homogenates from unfed ticks of all three developmental stages.

Identification of antigens in unfed tick homogenates by immunoblotting with post-infestation sera

Anti-larval, anti-nymphal and anti-adult sera identified several bands (in excess of 20) ranging from 23,000 to > 100,000 MW in all three homogenates. The major antigenic bands identified by each of the post-infestation sera are summarized in Table 5. Bands of 97,500 and 93,500 MW were weakly labelled in each of the three homogenates by all three antisera. An antigenic fraction of 70,000 MW was also present in all the homogenates when probed with anti-larval serum, strongly labelled in unfed nymphal homogenates (UNH), but weakly in unfed larval homogenates (ULH) and unfed adult homogenates (UAH). Anti-nymphal serum and anti-adult serum did not identify this band in ULH or UAH, but in UNH both sera labelled it strongly. The 70,000 MW band was, however, recognized in homogenates of partially fed and fully fed females by all three antisera (data not shown). A band of 55,000 MW was strongly labelled in each of the homogenates by anti-larval and anti-adult sera, but anti-nymphal serum labelled it only weakly. Bands of 51,500 and 40,000 MW were clearly labelled in all three homogenates by anti-larval and anti-adult sera, but anti-nymphal serum labelled these bands only weakly or not at all. The lower molecular weight species located at 36,500 and 23,000 were not recognized by anti-larval and anti-nymphal sera, but in all three homogenates probed with anti-adult serum both were present as intensely stained broad bands.

Table 5. Summary of major antigenic bands identified in crude unfed *R. appendiculatus* homogenates by polyspecific serum from *R. appendiculatus*-infested guinea-pigs

Antiserum to	Antigen source	Antigen MW							
		97,500	93,500	70,000	55,000	51,500	40,000	36,500	23,000
Larvae	ULH	±	±	±	++	+	+	−	−
	UNH	±	±	++	++	+	+	−	−
	UAH	±	±	±	++	+	±	−	−
Nymphs	ULH	±	±	−	±	±	−	−	−
	UNH	±	±	++	±	±	−	−	−
	UAH	±	±	−	±	±	−	−	−
Adults	ULH	±	±	−	++	+	+	+++	+++
	UNH	±	±	+++	++	+	+	+++	+++
	UAH	±	±	−	++	+	+	+++	+++

−, unlabelled; ±, weakly labelled; +, clearly labelled; ++, strongly labelled; +++, intensely labelled.

Table 6. Summary of major antigenic bands identified in crude unfed *R. appendiculatus* homogenates by polyspecific post-immunization sera from guinea-pigs

Antiserum to	Antigen source	Antigen MW									
		97,500	93,500	84,000	70,000	60,000	55,000	51,500	40,000	36,500	23,000
ULH	ULH	+++	+++	+++	++	+	±	+	−	−	−
	UNH	+++	+++	+++	+	+	±	+	−	−	−
	UAH	+++	+++	+++	±	+	±	+	−	−	−
UNH	ULH	+++	+++	+++	±	+	±	+	−	−	−
	UNH	+++	+++	+++	+	+	±	+	−	−	−
	UAH	+++	+++	+++	±	+	±	+	−	−	−
UAH	ULH	+++	+++	+++	++	+	±	+	+	−	−
	UNH	+++	+++	+++	++	+	±	+	+	−	−
	UAH	+++	+++	+++	++	+	±	+	+	−	−

−, unlabelled; ±, weakly labelled; +, clearly labelled; ++, strongly labelled; +++, intensely labelled.

Normal guinea-pig serum failed to identify any of these antigen bands.

Identification of antigens in unfed tick homogenates by immunoblotting with post-immunization sera

Many of the antigens identified by post-infestation sera were also identified by post-immunization sera, but there were some important differences also (Table 6). The high MW bands of 97,500 and 93,500 were intensely stained in all three homogenates by each of the post-immunization sera; so was an 84,000 MW antigen which was not recognized by post-infestation sera. A 60,000 MW species was also clearly labelled by the post-immunization sera but not by post-infestation sera. On the other hand, the 36,500 and 23,000 MW antigenic bands appeared to be specifically associated with feeding by adult ticks. Post-immunization sera did not recognize these in any of the homogenates, while post-adult infestation serum stained these two bands intensely in all three homogenates.

Normal guinea-pig serum did not recognize any of these antigenic bands.

DISCUSSION

Immunization with tick-derived antigens has considerable potential in controlling tick infestation of livestock. A variety of antigen sources have been used to immunize against different tick species (reviewed by Willadsen 1986; Brown, 1988). In most experiments with multi-host ticks, the challenge feed has been with the same stage from which the immunizing extracts were made. Recently Opdebeeck, Wong & Dobson (1989) have shown that cattle are protected against the 1-host *B. microplus* adult ticks by immunization with antigens purified from crude larval extracts to the same extent as with midgut extracts from partially fed female ticks. With *R. appendiculatus*, whole adult extracts (Mongi *et al.*, 1986), adult midgut extracts (Jongejan *et al.*, 1989) and female salivary gland extracts (Nyindo *et al.*, 1989) have been used successfully to immunize rabbits against *R. appendiculatus* adults.

Of the various criteria used for assessing the success of immunization, a significant reduction in engorged weight is considered as one of the most consistent (Rechav & Dauth, 1987). Our experiments confirm this and clearly show for the first time that unfed larvae and nymphs of *R. appendiculatus* provide an easily available and efficient source of antigen for immunization against adults of this species. In our experiments, the level of protective immunity achieved by individual animals even with the same homogenate varied, and in assessing the immunogenicity of putative vaccines, the level of protective immunity as well as the proportion of animals developing significant protection will have to be considered (Heller-Haupt *et al.*, 1989).

Nyindo *et al.* (1989) have pointed out that immunity following *R. appendiculatus* larval infestation was less potent in limiting subsequent feeding by nymphs and adults; they attributed this to the smaller amount of salivary secretion from the larvae compared to that from nymphs and adults and possibly to additional antigens in the salivary glands of adults. Although Shapiro, Voigt & Fujisaki (1986) reported that many of the antigens recognized in extracts of unfed adult *R. appendiculatus* by anti-adult tick serum were absent in extracts of unfed larval and unfed nymphal instars, our results suggest otherwise; so do the results of Nyindo *et al.* (1989) who showed that antisera to larval, nymphal and adult *R. appendiculatus* recognize many antigens in salivary gland extracts from adult *R. appendiculatus*. That there are shared antigens in the salivary glands of the different stages which are responsible for cross-immunity between the stages following infestation seems to be in no doubt. The close fit we obtained between the HPLC elution profiles of homogenates from all three developmental stages suggests that larvae, nymphs and adults have protein moeities of similar MW; furthermore, results from our immunoblotting experiments show that post-adult infestation serum has antibodies to several antigenic fractions found in homogenates of immature stages. The staining of two antigenic bands of 36,500 and 23,000 MW in all three homogenates by anti-adult serum, but not by anti-larval or anti-nymphal serum, suggests that they are either not presented or presented only minimally (not adequate enough to induce an immune response) to the host's immune system during the feeding period of immature stages. Recognition of the 70,000 MW band in homogenates of partially fed and fully fed

females by all three antisera suggests its association with the feeding process of adult ticks; why this band was recognized in unfed nymphal homogenates is not clear.

The recognition by post-immunization antisera of antigenic bands of 84,000 and 60,000 MW in whole body homogenates of unfed larvae, nymphs and adults of *R. appendiculatus* suggests that these bands may include 'concealed' antigens, similar to the ones described by Willadsen & Kemp (1988) for *B. microplus*, which are not normally encountered by the hosts during feeding by ticks but which may play a role in vaccination-induced immunity.

Identification and characterization of the protective antigen(s) of *R. appendiculatus* has not proceeded to the same stage as that of *B. microplus*, for which a glycoprotein of 89,000 MW with anisoelectric point of 5·1–5·6 and affinity for wheat germ lectin, isolated from fed female extracts, has been identified as one of the responsible antigens (Willadsen *et al.*, 1989). Whole tick homogenates contain antigens derived from a variety of sources, e.g. the cuticle and various internal organs and tissues including salivary glands and gut. Not all antigenic bands (each band may represent more than one antigen) recognized in homogenates of unfed immature stages by sera from our infested or immunized guinea-pigs may be responsible for protective immunity; but more than one, acting synergistically, may possibly be involved. The identity of the particular antigen(s) responsible for protective immunity to *R. appendiculatus*, can be confirmed only by purification of the different antigens to homogeneity and immunization of animals and challenge feeding by ticks, since some of the antigens or their epitopes may induce antibody production without conferring any protection.

Fivaz, Norval & Lawrence (1989) have suggested that host resistance to *R. appendiculatus* may be an important regulatory mechanism for establishing and maintaining enzootic stability in East Coast fever endemic areas. It is in this context, apart from tick infestation control, that immunization with tick-derived antigens should be considered.

ACKNOWLEDGMENTS

This work was funded in part by grants from the Overseas Development Administration of Great Britain and the Commission of the European Communities.

The biochemical and immunochemical studies reported in this paper formed part of a PhD thesis, submitted by P.K.E. Trinder to the University of London.

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